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Recombinant Technology

Expression of full-length immunoglobulins in *Escherichia coli*: rapid and efficient production of aglycosylated antibodies

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Abstract

Many research and clinical applications require large quantities of full-length antibodies with long circulating half-lives, and production of these complex multi-subunit proteins has in the past been restricted to eukaryotic hosts. In this report, we demonstrate that efficient secretion of heavy and light chains in a favorable ratio leads to the high-level expression and assembly of full-length IgGs in the *Escherichia coli* periplasm. The technology described offers a rapid, generally applicable and potentially inexpensive method for the production of full-length therapeutic antibodies, as verified by the expression of several humanized IgGs. One *E. coli*-derived antibody in particular, anti-tissue factor IgG1, has been thoroughly evaluated and has all of the expected properties of an aglycosylated antibody, including tight binding to antigen and the neonatal receptor. As predicted, the protein lacks binding to C1q and the FcγRI receptor, making it an ideal candidate for research purposes and therapeutic indications where effector functions are either not required or are actually detrimental. In addition, a limited chimpanzee study suggests that the *E. coli*-derived IgG1 retains the long circulating half-life of mammalian cell-derived antibodies. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: *E. coli* expression; Antibody production; Aglycosylated antibodies

1. Introduction

Monoclonal antibodies have recently become promising therapeutic proteins (King and Adair, 1999), in addition to their conventional use as research tools. These proteins can be targeted to almost any extracellular or cell surface protein and, through the

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simple act of binding, promote the blocking or activation of specific biochemical steps (Hori, 1991; Kim et al., 1993). Additionally, antibodies can couple their antigen to natural effector functions (Dyer et al., 1989; Reff et al., 1994) or perhaps provide another activity through conjugation (Hinman et al., 1993; Liu et al., 1996). One significant advantage of full-length IgGs is their long circulating half-life in mammals (Mariani and Strober, 1990), the result of both a large molecular size preventing clearance in the kidneys, and the ability of these proteins to avoid proteolysis in the endothelium by using a salvage pathway (Junghans, 1997). This pathway plays a major role in the slow clearance of IgGs and depends on binding of the immunoglobulin Fc-domain to the neonatal receptor (FcRn) (Simister and Mostov, 1989).

Full-length monoclonal antibodies have traditionally been produced in mammalian cell culture due to their original hybridoma source and due to the complexity of the molecule (King, 1998). More recently, transgenic plants offer an alternative route and potentially a more economical system of production (Conrad and Fiedler, 1998; Giddings et al., 2000). Generally, *Escherichia coli* is the host system of choice for the expression of antibody fragments such as Fvs, scFvs, Fabs or F(ab')₂s (Pluckthun et al., 1996; Kipriyanov and Little, 1999). These fragments can be made relatively quickly in large quantities with the retention of antigen binding activity. However, because antibody fragments lack the Fc domain, they do not bind the FcRn receptor and are cleared quickly; thus, they are only occasionally suitable as therapeutic proteins (Knight et al., 1995). Attempts to extend the heavy chain beyond the hinge have been successful with the expression of a C_H2-deleted antibody (Lo et al., 1992). The addition of the C_H3 domain promotes dimerization of the antigen binding arms; but the lack of the C_H2 voids any chance of the protein binding the FcRn receptor (Yasmeen et al., 1976). Full-length antibody chains can also be expressed in *E. coli* as insoluble aggregates and then refolded in vitro (Boss et al., 1984; Cabilly et al., 1984), but the complexity of this method limits its usefulness.

Although the assembly of full-length antibodies in *E. coli* has not been reported, two reasons suggested the feasibility of this approach. First, fully functional

antibody fragments through the hinge are routinely expressed as F(ab')₂s (Rodrigues et al., 1993; Koumenis et al., 2000), and second, the Fc fragment including the hinge has also been successfully produced as a dimer in *E. coli* (Kim et al., 1994). The aglycosylated Fc fragment bound to immobilized protein A, and displayed equivalent in vivo clearance to a full-length mammalian-derived glycosylated IgG. This data correlates well with previous studies showing that glycosylated and aglycosylated IgGs have equivalent in vitro FcRn binding and in vivo half-life in mammals (Tao and Morrison, 1989; Hobbs et al., 1992). Since both halves of a full-length antibody can be expressed and assembled independently in *E. coli*, the folded full-length molecule itself could likely be produced, with advances in technology.

This study demonstrates that a full-length anti-tissue factor (αTF) IgG1 (Presta et al., 2001) can be expressed at high levels in *E. coli*, produced in large-scale fermentors, and captured on immobilized protein A. In vitro, the purified protein binds tightly to both the antigen and the FcRn receptor and, as predicted, fails to bind C1q or FcγRI indicating a lack of effector functions. Additionally, the circulating half-life of the *E. coli*-derived IgG1, determined in chimpanzees, appears similar to glycosylated αTF IgG2 and IgG4 produced in mammalian cells.

2. Materials and methods

2.1. Plasmid construction

Expression cassettes were cloned into the framework of pBR322 (Sutcliffe, 1978) at the EcoRI site using standard methods (Chang et al., 1987). All constructions contained at least one *phoA* promoter (Kikuchi et al., 1981) and at least one lambda *t*₀ transcriptional terminator (Schöltissek and Grosse, 1987). Additionally, the STII signal sequence (Picken et al., 1983) or silent codon variants thereof (Simmons and Yansura, 1996) preceded the coding sequence for both light and heavy chains.

2.2. Small scale inductions and SDS-PAGE

Transformants from strain 33D3 (W3110 kan^R Δ*h*uA (Δ*tonA*) *ptr3* *lacIq* *lacL8* *ompTΔ* (*nmpc-fepE*)-

degP) were inoculated into 5 ml Luria–Bertani medium plus carbenicillin (50 ug/ml) and grown overnight at 30 °C. Each culture was then diluted (1:50 or 1:100) into, C.R.A.P. phosphate-limiting media (3.57 g (NH₄)₂SO₄, 0.71 g Na citrate–2H₂O, 1.07 g KCl, 5.36 g Yeast Extract (certified), 5.36 g HycaseSF-Sheffield, pH adjusted with KOH to 7.3, volume adjusted to 872 ml with deionized H₂O and autoclaved; cooled to 55 °C and supplemented with 110 ml 1 M MOPS pH 7.3, 11 ml 50% glucose, 7 ml 1 M MgSO₄ plus carbenicillin (50 ug/ml) and grown for approximately 24 h at 30 °C. Whole cell lysates from induced cultures (equivalent O.D. 600) were prepared and analyzed by SDS-PAGE (12% Tris–Glycine gels; NOVEX, San Diego, CA). Non-reduced samples were processed with 10 mM iodoacetic acid (Sigma I-2512) to prevent disulfide shuffling. The gels were then used for immunoblots.

2.3. Immunoblots

Following SDS-PAGE, samples from induced cultures were analyzed by immunoblot using standard methods. The protein bands bound by the anti-Fab antibody (peroxidase-conjugated goat IgG fraction to human IgG Fab; CAPPEL #55223) were visualized using an Amersham Pharmacia Biotech ECL detection kit and exposing the membrane to X-ray film.

2.4. N-terminal sequence analysis of small scale produced proteins

N-terminal sequence analysis of samples from small scale induced cultures were performed as described previously (Simmons and Yansura, 1996).

2.5. Fermentation

A 500 ml LB culture, grown for approximately 16 h at 30 °C with shaking, was used to inoculate a 10-l fermentor. The fermentor medium initially contained 8.9 mM glucose, 14.3 mM MgSO₄, 143 μM FeCl₃, 46 μM ZnSO₄, CuSO₄, H₃BO₃, 42 μM CoCl₂, NaMoO₄, MnSO₄, either 15–20 mg/l tetracycline or 50–70 mg/l ampicillin, 40.6 mM (NH₄)₂SO₄, 16 mM K₂HPO₄, 10 mM NaH₂PO₄, 3.6 mM sodium citrate, 12 mM KH₂PO₄, 29 g/l casein hydrolysate, and 14.3 g/l yeast

extract. Fermentations were performed at 30 °C, 650 rpm, and 2 vvm aeration. Concentrated ammonium hydroxide was added to maintain the pH at 7.0.

Following inoculation of the fermentor, the culture was grown to high cell densities using a computer-based algorithm to feed a concentrated glucose solution. When the culture reached approximately 20 OD₅₅₀, 1250 ml of a salt solution containing 12.5 g (NH₄)₂SO₄, 32.5 g K₂HPO₄, 16.25 g NaH₂PO₄, 2.5 g sodium citrate dihydrate, 18.75 g KH₂PO₄, 540 mg FeCl₃, 80mg ZnSO₄, CuSO₄, 70mg CoCl₂, NaMoO₄, 20 mg H₃BO₃, and 50 mg MnSO₄, was slowly fed to the culture. At a cell density of approximately 40 OD₅₅₀, 100 ml of 1 M magnesium sulfate was added. Fermentations were typically continued for 70–80 h.

2.6. Purification

Fermentation cell paste was diluted 1:5 (w/v) in 20 mM sodium phosphate pH 7.4, 0.14 M NaCl, then lysed using an M110Y microfluidizer (Microfluidics, Newton, MA). The cell lysate was clarified by centrifugation (4300 × g, 30 min) and polyethylene imine (BASF, Rensselaer, NY) was added to the supernatant to a final concentration of 0.2%, followed by a second centrifugation. After filtration (0.2 μm), the supernatant was applied to a Protein A affinity resin (Prosep A Millipore, Bedford, MA) and eluted using 0.1 M acetic acid pH 2.9. The Protein A pool was conditioned by the addition of urea to a final concentration of 2 M, adjusted to pH 5.5, then diluted with purified water and applied to SP Sepharose FF (Amersham Pharmacia Biotech, Uppsala, Sweden). The remaining process followed a previously described method for the purification of CHO-derived antibodies (Presta et al., 2001).

2.7. Chemical analyses

MALDI-TOF-MS, cation-exchange chromatography, amino acid and N-terminal analysis were performed on the purified *E. coli*-derived αTF using standard methods. Size exclusion chromatography was performed on purified samples using a 7.8 mm × 30 cm TosoHaas TSKgel G3000SWXL column. The column was run at room temperature with

a mobile phase of 50 mM sodium phosphate buffer, pH 6.1, at a flow rate of 0.5 ml/min. Protein was detected at 280 nm.

2.8. Antigen binding

Antigen binding was determined using an ELISA, with soluble tissue factor (TF) as the coat and peroxidase-conjugated goat anti-human F(ab')₂ (Jackson

ImmunoResearch, West Grove, PA) as the detecting antibody.

2.9. C1q, FcRn and FcγRI binding

The binding of purified *E. coli*-produced αTF to human C1q, human FcRn and the FcγRI receptor was determined using previously described assays (Idusogie et al., 2000; Shields et al., 2001).

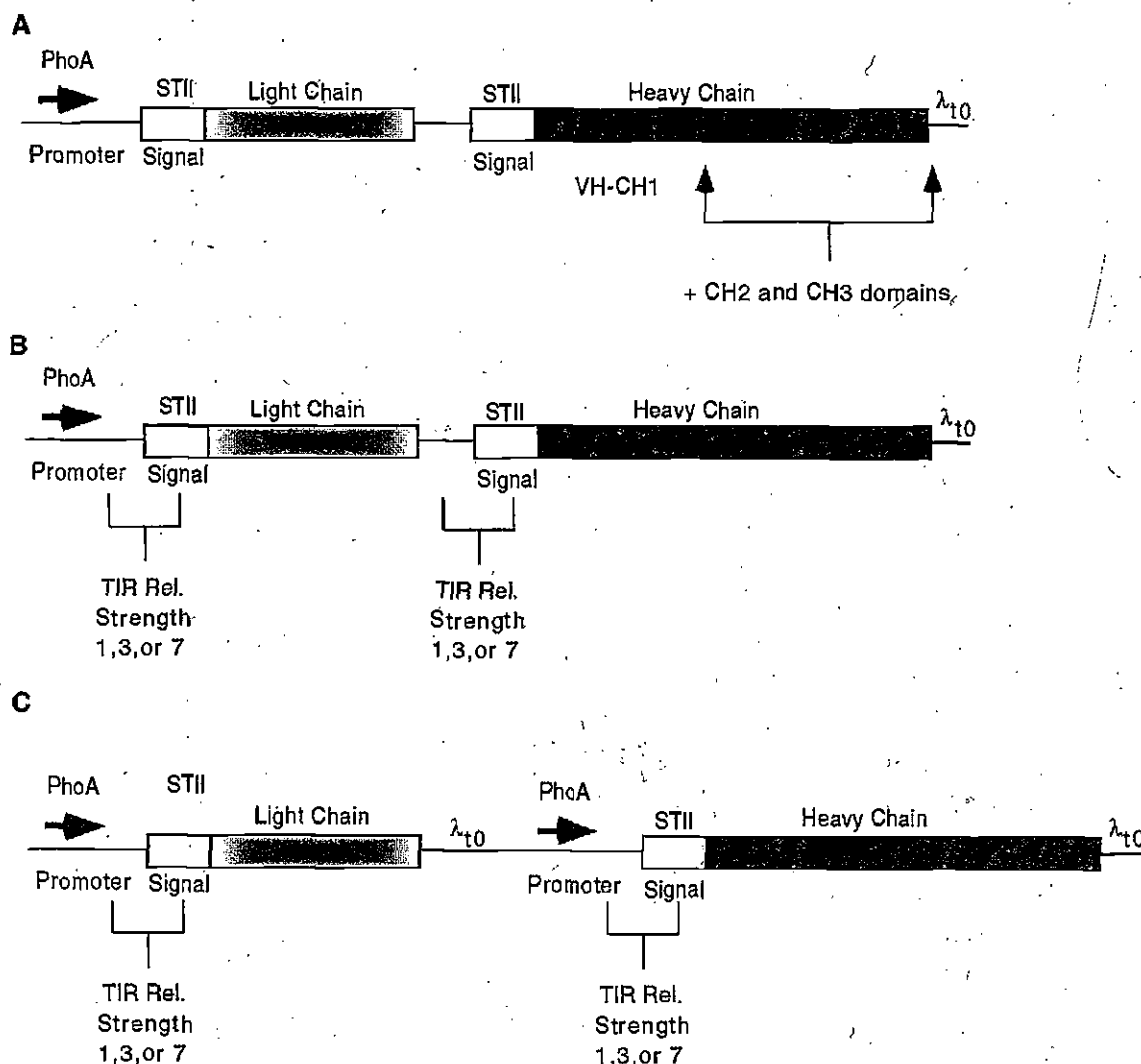


Fig. 1. Schematic of the expression plasmids. (A) polycistronic full-length expression plasmid derived from the published vector, pAK19 (Carter et al., 1992a,b). (B) Polycistronic full-length expression plasmids with varied TIR combinations. (C) Two cistron full-length antibody expression plasmids with varied TIR combinations.

2.10. Pharmacokinetics study

After testing negative for the presence of α TF antibodies, three chimpanzees each received a single IV bolus dose of α TF antibody (IgG1 *E. coli*, IgG2

CHO, or IgG4 CHO) at 0.10 mg/kg ($n=1$ for each antibody). Plasma samples were collected as follows: 30 and 15 min predose; 2, 15, 30 min; 1, 2, 3, 4, 6 and 12 h; 1, 2, 4, 7, 14, 21 and 28 days post IV bolus dose. Samples were assayed for α TF antibody (ATF) content

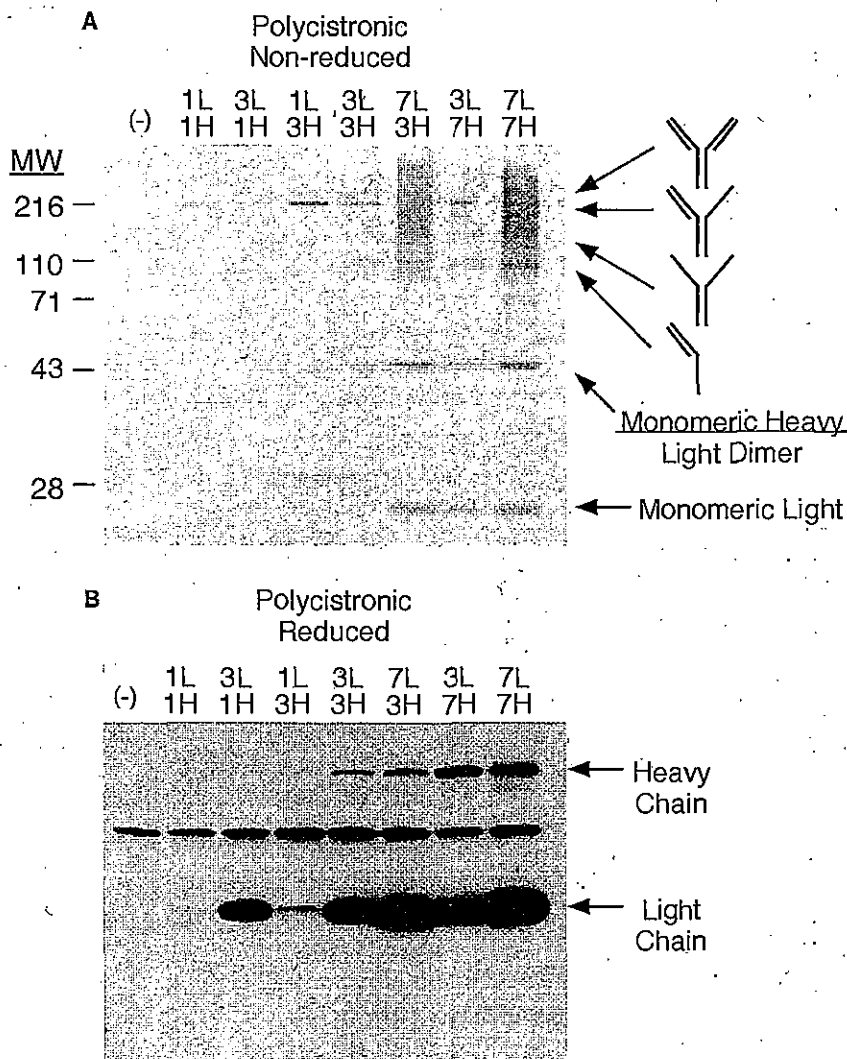


Fig. 2. Expression of full-length α TF IgG1 using the polycistronic vectors. Whole cell lysates were analyzed by SDS-PAGE immunoblot following induction. (A) Non-reduced samples. The top arrow indicates folded full-length IgG1 with all disulfide bonds formed. Monomeric light chain, and heavy chains, light chain dimer and other species, presumably lacking one or more disulfide bonds, are also indicated. The partially assembled species were verified by N-terminal analysis and mass spectrometry. (B) Reduced samples. The long exposure necessary to detect the heavy chain in the reduced samples also results in the detection of an *E. coli* protein. This band appears in all lanes, including the negative control. Listed above each lane is the relative TIR strength for light ("L") and heavy ("H") chain. (-): Induced cells harboring only the background vector, pBR322.

by ELISA, using TF as a coat and an anti-Fc monoclonal antibody as a detecting antibody. The limit of quantification was 0.102 µg/ml in chimpanzee plasma.

The plasma ATF versus time profiles were fit to a one-compartment elimination profile to determine volume of distribution, clearance and terminal half-life.

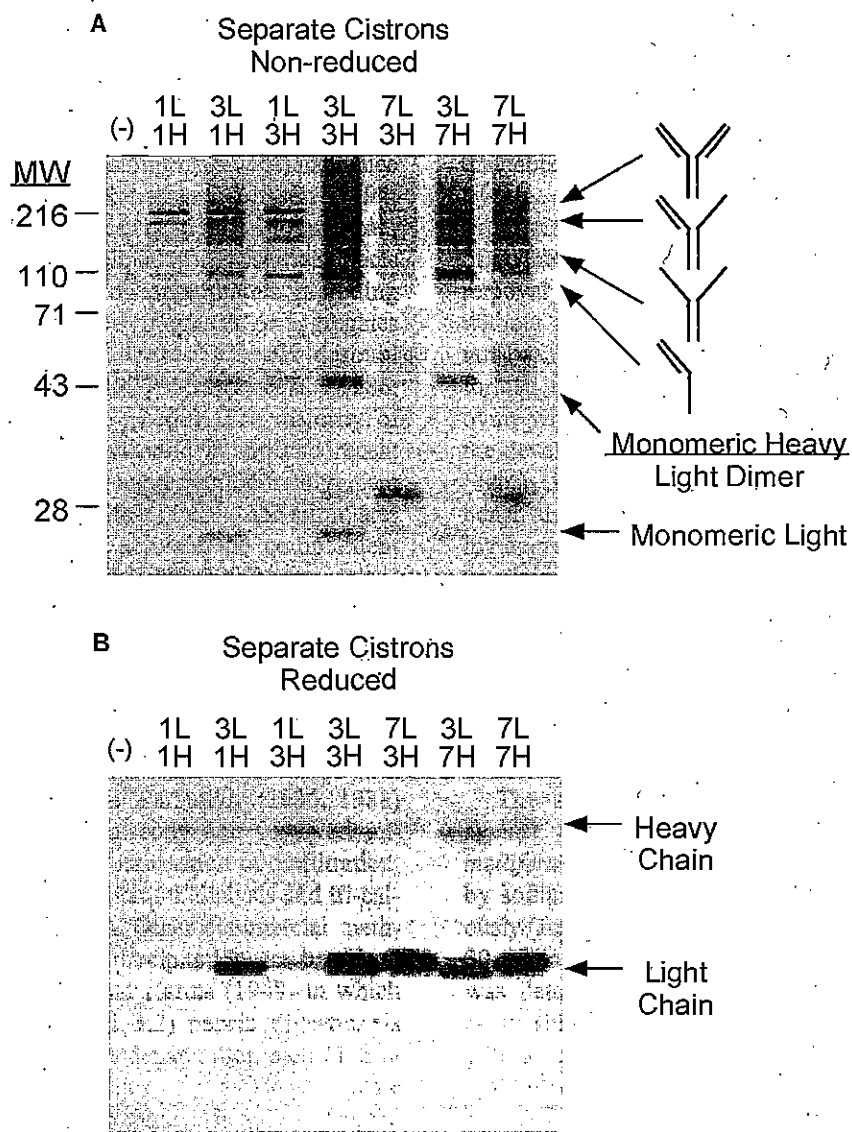


Fig. 3. Expression of full-length α TF IgG1 using the two cistron vectors. Whole cell lysates were analyzed by SDS-PAGE immunoblot following induction. (A) Non-reduced samples. The top arrow indicates folded full-length IgG1 with all disulfides formed. Monomeric light chain and heavy chains, light chain dimer and other species, presumably lacking one or more disulfide bonds, are also indicated. The partially assembled species were verified by N-terminal analysis and mass spectrometry. (B) Reduced samples. Since heavy chain was expressed at higher levels in these samples than in the polycistronic samples, only a short exposure was required when the immunoblot was developed. As a result, the additional *E. coli* band noted in Fig. 2 is not visible. Listed above each lane is the relative TIR strength for light ("L") and heavy ("H") chain. (-): Induced cells harboring only the background vector, pBR322.

3. Results

3.1. Attempts with a published vector

Initial attempts to produce full-length antibodies in *E. coli* focused on using the previously described Fab/ polycistronic expression vector pAK19 (Carter et al., 1992a). The V_L and V_H regions of the pAK19 Fab were replaced with those of the α TF antibody (Presta et al., 2001), and heavy chain was extended to encode the C_{H2} and C_{H3} domains of a human IgG1 immunoglobulin (Fig. 1A). The construct was transformed into a periplasmic protease deficient host, and grown in a phosphate-limiting media. Non-reduced whole cell lysates were analyzed by immunoblot using an anti-human Fab conjugated antibody as a probe; several bands were observed, including one having the molecular size of a full-length antibody. However, N-terminal amino acid analysis of the two chains from reduced samples revealed both processed and unprocessed light and heavy chain (data not shown).

This secretion problem was corrected by lowering translation levels using a series of translation initiation regions (TIRs) (Simmons and Yansura, 1996). Several constructs were prepared using different combinations of TIR strengths (Fig. 1B) for light and heavy chains, and the expression analyzed as previously described. Non-reduced whole cell lysates showed varying levels of putative full-length antibody as well as intermediate species (Fig. 2A). The optimal constructs for producing full-length antibody were 1-light 3-heavy followed by 3-light 7-heavy. Additionally, samples from the 7-light 3-heavy and 7-light 7-heavy constructs showed considerable smearing, indicative of antibody aggregation. Analysis of the reduced samples revealed a large excess of light chain over heavy for all constructs, even taking into consideration that the anti-human Fab antibody detects light chain more readily than heavy chain (Fig. 2B). Accumulation of precursor light chain was also apparent for the 7-light 7-heavy construct. Notably, the two constructs producing the greatest accumulation of full-length antibody

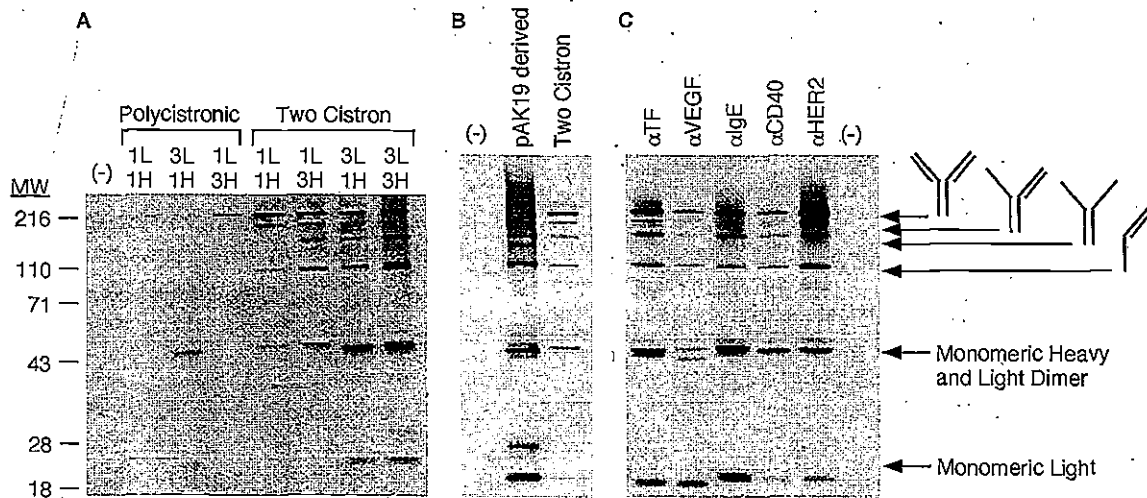


Fig. 4. Comparison of the two cistron vs. the polycistronic systems. Non-reduced whole cell lysates were analyzed by SDS-PAGE immunoblot following induction. (A) Comparison of α TF IgG1 expression using the polycistronic vectors and the two cistron vectors. Listed above each lane is the relative TIR strength for light ("L") and heavy ("H") chain. (-): Induced cells harboring only the background vector, pBR322. (B) Comparison of α TF IgG1 expression using the pAK19-derived polycistronic vector (Carter et al., 1992a) and the two cistron vector with TIR relative strengths of 1-light 1-heavy. (C) Expression of additional antibodies using the two cistron vector with relative TIR strengths of 1-light 1-heavy. The top arrow indicates folded full-length IgG1 with all disulfides formed. Monomeric light chain and heavy chains, light chain dimer and other species, presumably lacking one or more disulfide bonds, are also indicated. The partially assembled species were verified by N-terminal analysis and mass spectrometry.

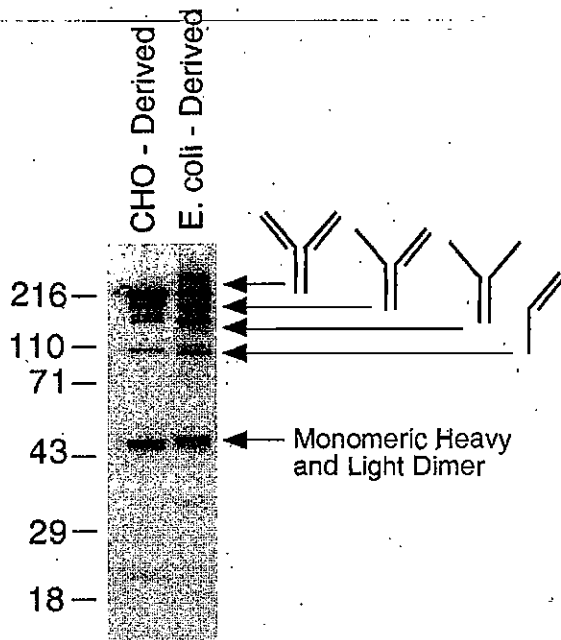


Fig. 5. Immunoblot comparison of *E. coli* vs. CHO-derived α TF IgG1. Non-reduced samples were analyzed by SDS-PAGE immunoblot. Shown are whole cell lysates for the *E. coli* sample and tissue culture supernatant for the CHO sample.

displayed the lowest light to heavy ratio, while the two constructs producing significant aggregation appeared to correlate with a large excess of light chain and the accumulation of precursor chains.

3.2. Two cistron system

The data from the polycistronic constructs suggested that lower light to heavy ratios might improve the accumulation of folded full-length antibody. However, the polycistronic design makes it difficult to control each chain individually since the translation of the second gene (heavy) is dependent on the light chain TIR, the translational coupling between the two genes, and finally, the heavy chain TIR. Placing each chain under the control of its own promoter addresses this problem. In a two cistron design, translational initiation of each chain is only dependent on its own TIR and the ratio of light to heavy chain can be more readily adjusted.

A series of constructs was prepared to determine the optimal TIR strengths for light and heavy chain

using a two promoter system (Fig. 1C). The expression from these plasmids was then analyzed as previously described (Fig. 3A and B). Non-reduced samples of whole cell lysates produced similar antibody related bands to those observed from the polycistronic constructs, including a putative full-length species, with the optimal TIR combinations being 1-light 1-heavy and 1-light 3-heavy. Although other combinations displayed a full-length antibody band under non-reduced conditions, significant smearing caused by antibody aggregation was also apparent. Reduced samples showed a more even distribution of light and heavy chains than that of the polycistronic series. In particular, the optimal constructs for producing full-length antibody displayed almost equal band intensities for light and heavy chains, while the remainder produced excess light, a factor associated with aggregation under non-reduced conditions. Addi-

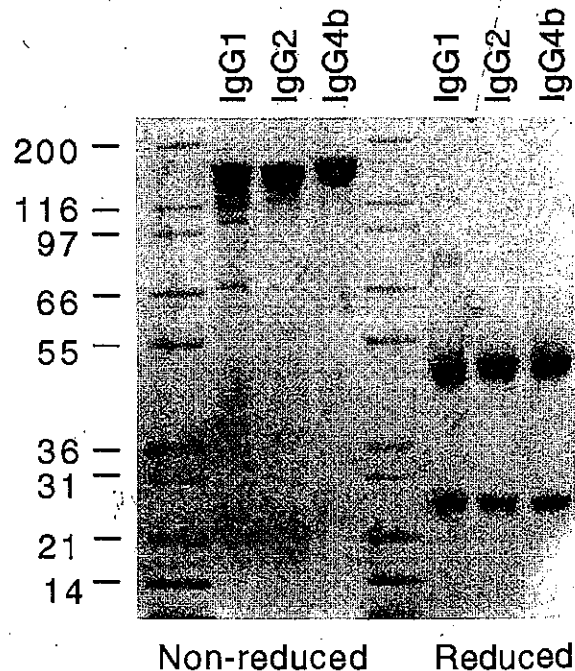


Fig. 6. SDS-PAGE analysis of purified protein. Following purification, non-reduced and reduced samples of the *E. coli*-derived IgG1 and the mammalian-produced IgG2 and IgG4b were analyzed on a NOVEX NuPAGE 4–12% gel using MOPS buffer. The gel was stained with Coomassie blue following electrophoresis. CHO-derived IgG4b is an engineered variant of IgG4 (Ser H241 Pro in the hinge region) for improved formation of inter-heavy chain disulfide bonds (Presta et al., 2001).

tionally, unprocessed light and heavy chains were observed for some constructs (3-light 3-heavy, 7-light 3-heavy and 7-light 7-heavy).

3.3. Comparison of the two cistron versus the polycistronic systems

A direct comparison of the separate cistron and polycistronic systems illustrates the advantage of improved control over the light to heavy chain ratio. First, non-reduced samples of whole cell lysates were prepared using plasmids with optimized TIR constructions from each system. The results demonstrate that even when the polycistronic TIRs are adjusted, the separate cistron constructs still produce considerably more full-length antibody than their polycistronic counterparts (Fig. 4A).

A second analysis involved a comparison of non-reduced whole cell lysate samples from the two cistron plasmid (1-light 1-heavy) with the construct derived from the published Fab' vector (Fig. 4B). An

induced full-length antibody band is observed in both samples; however, the expression level increases dramatically using the two cistron plasmid and the smearing associated with antibody aggregation is significantly reduced.

This two cistron technology was further tested by constructing expression plasmids for additional antibodies. As illustrated in Fig. 4C, antibodies against VEGF (higher affinity variant of the published humanized antibody; Presta et al., 1997), IgE (Presta et al., 1993), CD40 (humanized version of the murine antibody; Francisco et al., 2000) and HER-2 (Carter et al., 1992b) were also successfully expressed.

3.4. Scale-up and purification

An appropriate sized band on the immunoblots suggested full-length IgG1 expression and assembly. However, further analysis was required for a conclusive identification of this product. Initially, an immunoblot comparison was done using the *E. coli*-derived

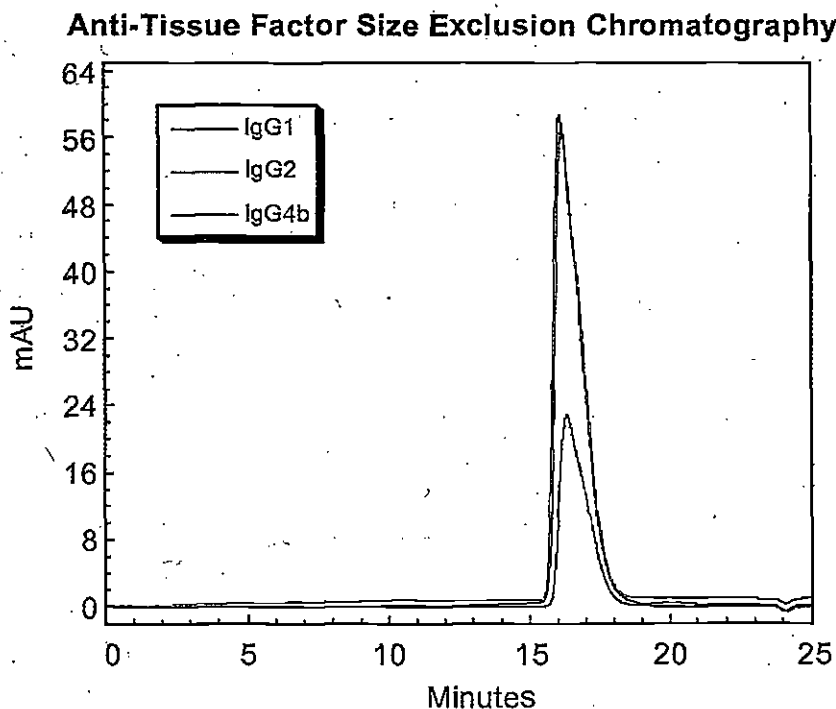


Fig. 7. Size exclusion chromatography of purified protein. Following purification, *E. coli*-derived IgG1 and the mammalian-produced IgG2 and IgG4b were analyzed by size exclusion chromatography. CHO-derived IgG4b is an engineered variant of IgG4 (Ser H241 Pro in the hinge region) for improved formation of inter-heavy chain disulfide bonds (Presta et al., 2001).

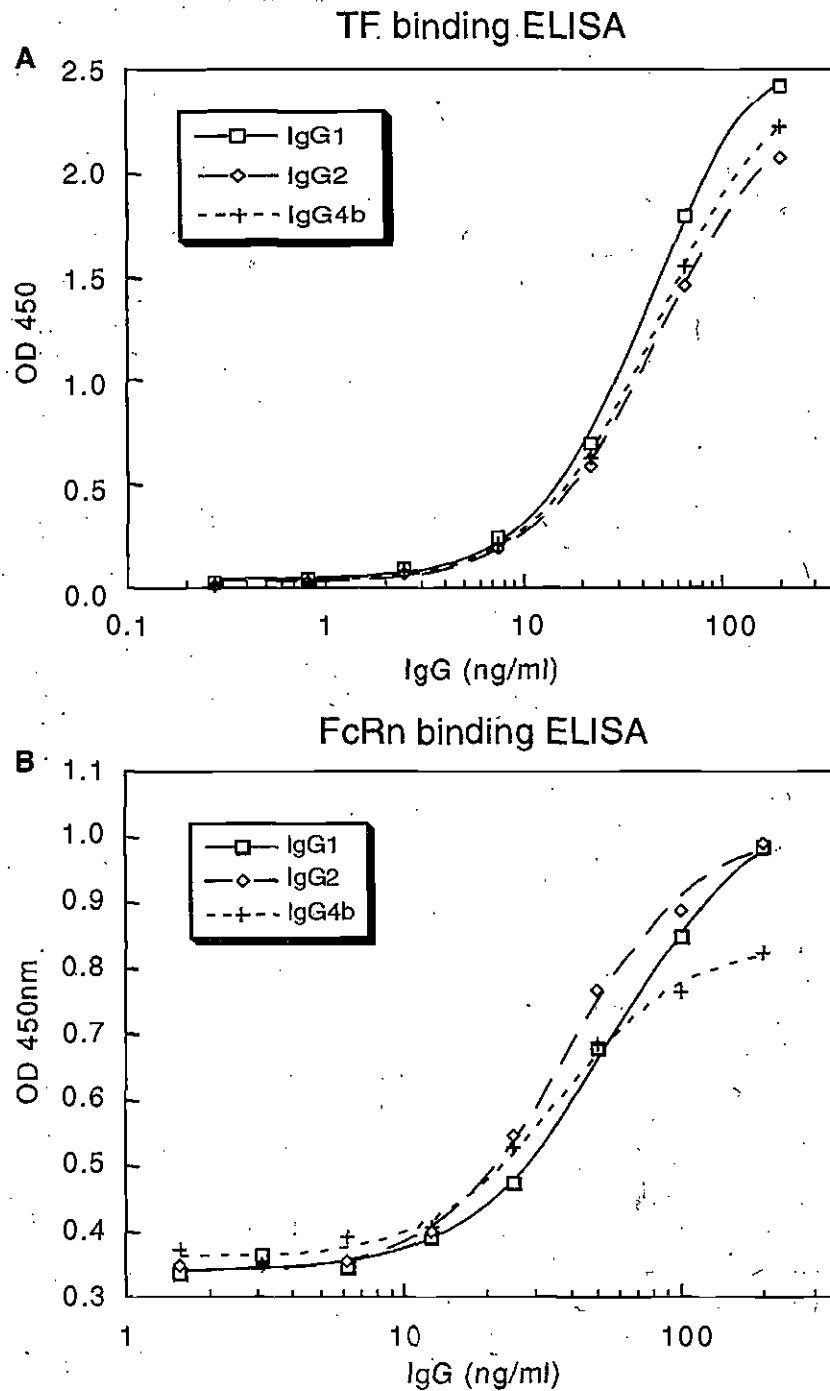


Fig. 8. Tissue factor and neonatal receptor binding assays. (A) Binding of purified *E. coli* derived α TF IgG1 and mammalian produced control α TF antibodies to soluble tissue factor. CHO-derived IgG4b is an engineered variant of IgG4 (Ser H241 Pro in the hinge region) for improved formation of inter-heavy chain disulfide bonds (Presta et al., 2001). (B) Binding of the α TF antibodies to the neonatal receptor, FcRn.

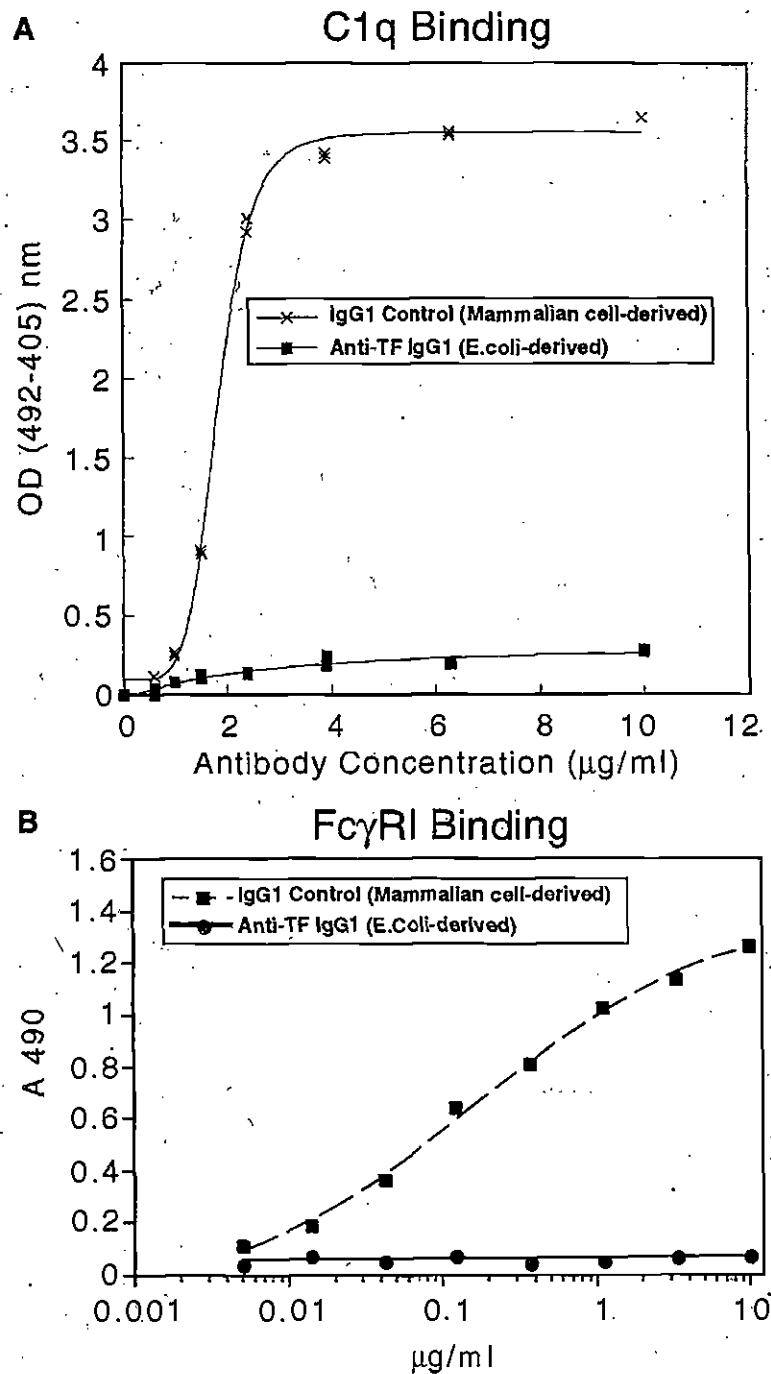


Fig. 9. C1q and FcγRI binding assays. (A) Binding of a mammalian-produced control antibody, Rituxan (Idusogie et al., 2000), to C1q. No binding was detected for the *E. coli*-derived αTF IgG1. (B) Binding of mammalian-produced αTF IgG1 to FcγRI. No binding was detected for the *E. coli*-derived αTF IgG1.

sample and an α TF IgG1 standard secreted by mammalian cells. As shown in Fig. 5, the putative fully assembled antibody from *E. coli* co-migrates with the completely assembled product from mammalian cells. Notably, the samples also show similar banding patterns, demonstrating that the presence of lower molecular weight species are not due to issues specific to *E. coli* antibody production. While the comparison with the CHO standard provided additional support that this *E. coli* system was producing fully assembled antibody, the next step in the process was to extensively test purified material obtained from fermentation paste.

Fermentations were performed using the best polycistronic (1-light 3-heavy) and separate cistron (1-light 1-heavy) constructs. The large-scale production of full-length antibody was analyzed using the AME5-RP assay (Battersby, in preparation). This quantitative assay, calibrated with an α TF aglycosylated IgG1, utilizes an immobilized Fab to first capture protein complexes possessing a light chain. A reversed phase separation of the captured species follows, distinguishing the majority of the partially assembled products from a peak containing primarily fully folded IgG1. In contrast, data from Protein G or Protein A assays include quantitation of the partially assembled species; thus, the results from the AME5-RP assay provide a more accurate calculation of yield. Samples from the polycistronic fermentation gave titers of 18 mg/l, while the separate cistron construct resulted in titers as high as 134 mg/l using the same host strain, and 156 mg/l in an alternate strain.

Following fermentation, antibody from the soluble cell lysate, where the majority of folded full-length antibody is found, was captured on protein A resin, and further purified by chromatography using SP sepharose followed by Q sepharose. An SDS-PAGE gel of this material is shown in Fig. 6 along with the purified α TF IgG2 and IgG4b produced in mammalian cells (Presta et al., 2001).

3.5. Characterization of the full-length IgG1

The purified α TF IgG1 was then characterized in terms of its chemical and biochemical properties. Size exclusion chromatography (Fig. 7) of the *E. coli*-derived IgG1 and the mammalian-produced IgG2 and IgG4b each showed the expected molecular weight

for a full-length intact antibody. Additionally, mass spectrometry, cation exchange chromatography, amino acid analysis and N-terminal amino acid analysis all produced the expected results. The purified *E. coli* IgG1 bound tissue factor and the FcRn receptor with similar affinity to that of α TF IgG2 and IgG4 produced in mammalian cells (Fig. 8A and B). However, as expected, the *E. coli*-produced aglycosylated IgG1 failed to bind C1q and the Fc γ RI receptor while mammalian-derived IgG1 control antibodies bound these proteins tightly (Fig. 9A and B), suggesting that the *E. coli*-derived IgG1 lacks effector functions.

3.6. Pharmacokinetic properties in chimpanzees

The *E. coli*-derived full-length α TF was subject to a single IV bolus dose chimpanzee pharmacokinetic (PK) study, along with control α TF produced from mammalian cells. No obvious differences in PK parameter estimates between the antibody made in *E. coli* and the antibodies made in mammalian cells were observed (Fig. 10). While it should be noted that

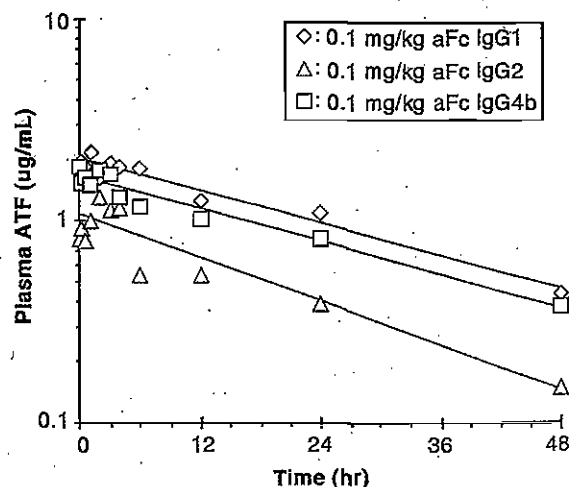


Fig. 10. Pharmacokinetic study of the purified *E. coli*-derived α TF IgG1. The pharmacokinetic properties of the *E. coli*-derived α TF IgG1 and mammalian produced control antibodies were determined in chimpanzees. The results represent a single experiment for each antibody. Calculated $T_{1/2}$ (day) values: 0.94 for *E. coli*-derived IgG1; 0.93 for CHO-derived IgG4b (Ser H241 Pro-mutation in hinge region) (Presta et al., 2001); 0.69 for CHO-derived IgG2 (Presta et al., 2001).

this experiment involved only a single chimpanzee for each antibody, these results are consistent with published data comparing glycosylated versus aglycosylated IgG1 antibodies (Tao and Morrison, 1989; Dorai et al., 1991; Hand et al., 1992; Baez et al., 2000). The authors in each study produced aglycosylated antibodies by genetic alteration, as opposed to tunicamycin treatment, and reported that the pharmacokinetic properties remained comparable to those of the glycosylated antibody.

4. Discussion

This report demonstrates that aglycosylated full-length antibodies can be successfully expressed and assembled in *E. coli* using a two cistron system with optimized light and heavy chain translation levels. Applying this approach, we were able to overcome the two primary problems identified using the traditional polycistronic design. First, simply adding the C_H2 and C_H3 domains to a typical vector described in the literature for the production of antibody fragments leads to inefficient secretion. Adjustments of the TIRs eliminated the secretory block; nonetheless, the expression level of heavy chain was still low in comparison to the level of light chain, highlighting the second problem associated with a polycistronic design. This design created an inherent gene expression imbalance due to inadequate translational control over the second gene on the mRNA. Thus, these constructs produce an overwhelming excess of light chain, the first gene on the message, relative to heavy chain, the second gene. However, with the two promoter system and optimized TIRs, heavy chain is efficiently secreted and a more desirable ratio of light to heavy chain is obtained, allowing the individual chains to then assemble into a correctly folded tetrameric complex. Interestingly, the ratio with the new system was closer to 1, corresponding to the light to heavy chain ratio noted in mammalian cell hosts (Scharff and Laskov, 1970; Bibila and Flickinger, 1991). This approach has proven successful for the expression in *E. coli* of several full-length antibodies including α TF, α VEGF, α CD40, α IgE, and α HER2.

Moreover, this technology is not limited to small-scale experimental work. The titers of *E. coli*-derived α TF produced in fermentors were approximately

130–150 mg/l of assembled antibody. Following purification, the IgG1 underwent a thorough chemical analysis and all results were consistent with expected properties. In addition, the *E. coli*-produced antibody strongly bound the antigen and the neonatal receptor; yet, as predicted, no binding was detected to C1q or the Fc γ RI receptor. Importantly, the *E. coli*-derived antibody appears to retain the desirable long half-life of a full-length antibody as demonstrated by a limited chimpanzee pharmacokinetic study.

Production of antibodies in *E. coli* offers several advantages over existing methods. First, ease of scale up and shorter fermentation run times using *E. coli* reduces the duration from plasmid construction to purified protein. A process that routinely takes several months in mammalian systems can take as little as one month with *E. coli*. Second, fermentation capital costs for therapeutic proteins are lower for bacterial production systems. And, third, the data suggest that *E. coli*-produced antibodies lack effector functions, a property desirable in many therapeutic indications that only require a blocking function by the antibody (Presta et al., 1993; Thompson et al., 1999; Armour et al., 1999; Presta et al., 2001). Indeed, for some indications, effector functions may have a deleterious impact on treatment (Isaacs et al., 1996; Friend et al., 1999; Reddy et al., 2000). Thus, *E. coli* offers a new route for the production of many therapeutic full-length antibodies and, in the less common scenario where killing of the target cell is desired, other techniques, such as coupling to a chemical toxin, could be employed.

The technology described serves as a significant step in the process of efficiently producing full-length aglycosylated antibodies in *E. coli*. The yields are presently high enough to routinely and rapidly supply material for research purposes, and the use of these proteins as therapeutic agents should be forthcoming with further increases in titer.

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